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AD NUMBER
AD595689

CLASSIFICATION CHANGES
TO: unclassified
FROM: confidential

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FEB 9 1972

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AN INVESTIGATION INTO THE USE OF IMMOBILISED CHOLINESTERASE FOR THE AUTOMATIC DETECTION OF NERVE AGENTS [C]

by

J. Lambert   E.J. Olsen   C. Stratford and G. Williams
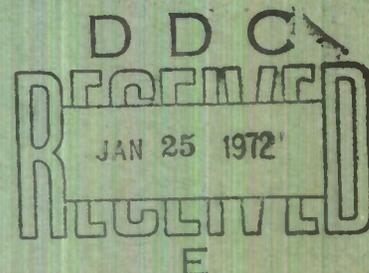
Technical Paper No. 57
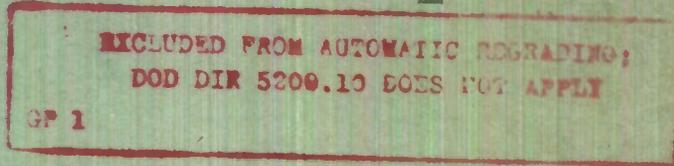
May 1971

Chemical Defence Establishment, Porton Down, Salisbury, Wilts.

Cat #115560

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Nerve agents.
Chemical agent detection

I Lambert, J.
II AD 595689.
III Cholinesterase.
IV Gt. Brit. - Nerve agents.

CONFIDENTIAL

C.D.E. TECHNICAL PAPER NO. 57
DATE: MAY 1971

AN INVESTIGATION INTO THE USE OF IMMOBILISED CHOLINESTERASE FOR THE AUTOMATIC DETECTION OF NERVE AGENTS

by

J. LAMBERT, E.J. OLSEN. C. STRATFORD, AND G. WILLIAMS

SUMMARY

The automatic detection of nerve agent vapours using cholinesterase covalently bonded to a modified polymethacrylate ion exchange resin and incorporated into an absorbent permeable paper, is described. The system responds to G-agent concentrations as low as 0.005 mg/m^3 in under 5 minutes and to high agent concentrations ($\geqslant$ 10 mg/m^3) in 5 - 8 seconds.

The electrochemical method used to monitor the inhibition of enzymic activity has been studied and the problems associated with electrode design and the ability to achieve a stable operating baseline are discussed.

A rapid photometric method for the assay of insoluble cholinesterase is described.

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INTRODUCTION

The ability of the organophosphorus nerve agents to inhibit the hydrolytic activity of the enzyme cholinesterase can be exploited to provide a specific, rapid and extremely sensitive method for their detection. During the past decade considerable efforts have been made, both in this country and abroad, to utilise this principle for the purpose of automatic vapour detection, where sensitivity and speed of response are of vital importance. In such a detector, a solution of cholinesterase in a continuous flow system is exposed to the atmosphere and its hydrolytic activity is monitored by the use of a suitable substrate. Both colorimetric (1) and electrometric (2) methods have been used for the determination of the hydrolysis products but, in each case, a major disadvantage has been the consumption of excessive quantities of the enzyme.

One method of solving the problem of enzyme consumption is to use an insolubilised form of the enzyme so that the same sample can be used continuously for several hours. The feasibility of this approach has been demonstrated by recent investigations in the US (3) using cholinesterase which is physically entrapped in a starch-coated polyurethane foam. This type of immobilisation is not totally satisfactory however, since, in the presence of solution, it has proved difficult to obtain permanent retention of the full enzymic activity. It is the purpose of the present paper to show that the latter problem can be successfully overcome by using a chemically

insolubilised enzyme, where the cholinesterase is covalently bonded to a modified polymethacrylate ion exchange resin (4).

In order to examine the feasibility of using this enzyme in a detector, the polymer is incorporated into an absorbent permeable paper mat which is placed between two platinum electrodes in an electrochemical cell. Air and substrate solution, butyrylthiocholine methanesulphonate, are drawn continuously through the paper and a small constant current is applied to the electrodes. Under clean air conditions, the cholinesterase hydrolyses the substrate and a constant potential difference across the electrodes is observed, due to the anodic oxidation of thiocholine to choline disulphide (5):

$$RSCOR' \xrightarrow{\text{ChE/H}_2\text{O}} RSH + R'COOH$$

$$RSH \rightleftharpoons RS^- + H^+$$

$$2RS^- \longrightarrow RSSR + 2e$$
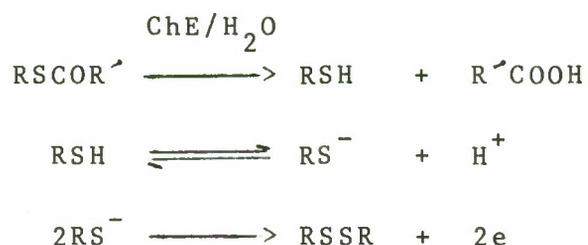
where $R = (CH_3)_3\overset{+}{N}CH_2CH_2$

$R' = C_3H_7$

In the presence of nerve agents the cholinesterase is wholly or partially inhibited, the concentration of thiocholine is reduced and the potential difference across the electrodes increases. Following complete inhibition of the enzyme the voltage rises indefinitely but can be limited to a value approximately 250 mV higher than the thiocholine oxidation potential by incorporating an equimolar amount of potassium iodide into the substrate solution. The anode is now considered (5) to measure the oxidation of iodide ion:

$$2I^- \longrightarrow I_2 + 2e$$

EXPERIMENTAL

Enzyme Paper

Enzyme polymer (4) was mixed with paper pulp and formed into paper using a Standard Pulp Evaluation Machine. The paper was approximately 1 mm thick and it was found that maximum permeability could be achieved by using 65 - 70% by weight of enzyme polymer of 100 - 200 BSS particle size in the pulp. The enzymic activity of the paper was controlled by the activity of the polymer (10 - 30 IU/g). (1 IU represents the quantity of enzyme required to hydrolyse 1μ mole of acetylcholine chloride per minute at pH 7.4 and 25°C).

Enzyme Pads

For most of the work described, small discs, 7.6 mm in diameter, were cut from the stock paper as required, each disc containing 0.2 IU of cholinesterase. Preliminary experiments showed that larger discs, still containing 0.2 IU of enzyme, were less satisfactory.

It was not possible to measure the cholinesterase activity of the enzyme-polymer by the usual method of monitoring the rate of acid production during the hydrolysis of an ester substrate because of the difficulty which was experienced in neutralising all the free acid groups present in the polymer. Known photometric methods, such as that described by Ellman (7), require lengthy procedures and so a simple indirect method of assay was developed (Appendix) where the activity of the enzyme polymer is expressed in terms of the known activity of a solution of cholinesterase. The method has proved particularly useful for the comparative measurements required in detector evaluation work and the activity values obtained have been in good agreement with those given by the Ellman method.

Substrate Solution

Substrate solution was made up daily and contained butyryl-thiocholine methanesulphonate (2×10^{-4}M), potassium iodide (2×10^{-4}M) and ethylene glycol (1%) in 'Tris' buffer (0.1M), pH 7.4. The substrate concentration was determined from the known optimum value for the cholinesterase-catalysed hydrolysis of butyrylthiocholine iodide (6).

Thiol Determination

The quantity of thiocholine produced by enzymic hydrolysis during operation of the detector was determined by colorimetric analysis of the cell effluent using 5.5'-dithiobis-(2-nitrobenzoic acid)(7).

The Detector Cell

A diagram of a typical detector cell, constructed in glass, is given in Figure 1. The enzyme pad rests in a rubber ring between two platinum electrodes and the whole is clamped between the two halves of the cell body to form an air and liquid-tight seal. Air (1 l/min) enters the cell via a 1 mm diameter PTFE tube and substrate solution (0.7 ml/min) through a 0.42 mm diameter stainless steel needle. The two delivery tubes are arranged concentrically above the centre of the enzyme pad with the tip of the substrate delivery needle being exactly 5 mm above the surface of the top electrode. This arrangement ensures that a steady stream of fine droplets, which is essential for the satisfactory operation of the detector, falls onto the top electrode. A first engineered version of the detector cell, constructed in PTFE and giving easier location of the electrode assembly, is illustrated in Figure 2.

Electrodes

Electrodes were bright platinum and were cleaned by heating to redness in an alcohol flame before use. Anodes were constructed from 0.025 mm thick perforated foil (Figure 3a) or 0.06 mm thick wire gauze (1024 mesh per square cm), while cathodes were perforated foil or 0.056 mm diameter wire formed into a 2 mm diameter loop (Figure 3b).

Electrical System

A small constant current was applied to the electrodes using a 22 V dry cell in series with a variable high resistance. The potential difference across the electrodes was measured with a high input impedance potentiometric strip chart recorder.

RESULTS

The response times quoted are arbitrary values and refer to the time taken for the signal voltage to exceed a value of five times the baseline 'noise'.

Optimum Applied Current

Baseline voltages for hydrolysed and unhydrolysed substrate were recorded for a series of applied currents in the range 0.3 to 2.0 μA, using perforated platinum foil for both electrodes. The results obtained are shown in Table 1 and depicted graphically in Figure 4, where it can be seen that the maximum signal that can be expected occurs when the applied current is 0.5 μA.

TABLE 1

VARIATION OF APPLIED CURRENT

Current	Baseline Voltage			Pad Inhibition	
(μA)	Iodide (mV)	Thiol (mV)	Theoretical Signal (mV)	Signal (mV)	Response Time (sec)
0.30	430	230	200		
0.50	500	275	225	105	14
0.75	535	345	190		
1.00	565	390	175	110	7
1.25	590	425	165	110	5
1.50	600	460	140	100	5
1.75	615	480	135		
2.00	625	500	125	100	5

In order to optimise the applied current with respect to speed of response as well as signal magnitude, hydrolysed substrate baselines were recorded for selected current values and the enzyme pads were completely inhibited with GB vapour (concentration approximately 10 mg/m^3). The response times observed are listed in Table 1 and it can be seen that the quickest responses were obtained when the applied current was in the range 1.25 to 2.0 μA. Since the optimum current values in terms of speed of response and maximum theoretical signal did not coincide, a compromise figure of 1.3 μA was selected as the optimum current and this value was used for all subsequent experiments.

Response to Agents

The detector was allowed to run for a period of one hour under clean air conditions and was then exposed to a known concentration of agent vapour in a 100 m^3 test chamber. Table 2 shows the results obtained with a variety of G-agents using perforated foil electrodes.

TABLE 2

RESPONSE TO G-AGENTS

Agent Concentration (mg/m^3)	Response Time (sec)				Ultimate Signal (mV)			
	GA	GB	GD	GF	GA	GB	GD	GF
10	8	5	7	6	190	200	220	140
1	15	13	10	12	150	170	100	130
0.1	36	32	23	20	180	280	200	120
0.01	195	66	63	54	160	180	100	160
0.005	275	112	90	92	80	130	110	140

Baseline Studies

One of the difficulties encountered in obtaining a satisfactory performance from the detector has been the problem of baseline drift and, in particular, the extremely high rate of voltage change experienced during the first half hour of operation. A typical hydrolysed substrate baseline, using perforated foil electrodes, drifted at a rate of 5.6 mV/min for the first half hour but at a rate of only 0.6 mV/min, thereafter.

There are two possible causes of baseline drift, (i) a variation in the amount of thiocholine produced by enzymic hydrolysis, (ii) a change in the degree of polarisation of the electrodes. Each of these possibilities was examined in turn.

(i) Thiocholine Concentration

Analysis of the liquid effluent from the detector cell revealed that the thiocholine concentration varied from 2.8×10^{-5} M to 3.6×10^{-5} M (14 to 18% of the original substrate concentration) during a typical twelve hour running period. To ascertain to what extent this variation affected the hydrolysed substrate baseline, known concentrations of thiocholine methane-sulphonate in 0.1M - 'Tris' buffer, pH 7.4, containing potassium iodide (2×10^{-4} M), were passed through a detector cell containing a pad possessing zero enzymic activity. The baseline voltages recorded for a range of thiocholine concentrations from 6×10^{-6} M to 1.6×10^{-4} M are listed in Table 3 and illustrated graphically in Figure 5.

TABLE 3

EFFECT OF THIOCHOLINE CONCENTRATION UPON BASELINE VOLTAGE

Thiocholine Concentration (μmole/l.)	Baseline Voltage (mV)
0	680
6	650
10	637
20	390
30	378
40	360
60	350
100	340
160	333

Examination of Figure 5 reveals that, although the detector is operating very near to the critical part of the response curve, the change in thiocholine concentration from 2.8×10^{-5} M to 3.6×10^{-5} M produces only a 10 mV change in baseline voltage. It was concluded that the variation in the amount of thiocholine produced by enzymic hydrolysis was not a significant contributor to the observed baseline drift.

(ii) Electrodes

Measurement of the anode and cathode potentials relative to a standard calomel electrode in a three electrode system, indicated that 87% of the observed increase in voltage on changing from hydrolysed to

unhydrolysed substrate occurred at the anode. Baseline drift, however, was almost equally apparent at both electrodes (Table 4; Figure 6) although the cathode took longer than the anode to achieve a stable potential.

TABLE 4

EFFECT OF ANODE AND CATHODE UPON BASELINE DRIFT

Running Time (min)	Drift in Electrode Potential (mV)		Total Baseline Drift (mV)
	Anode	Cathode	
0	0	0	0
2	40	55	95
5	68	80	148
10	100	119	219
15	112	140	252
20	120	155	275
30	124	170	294
40	126	179	305

The fact that the majority of the observed baseline drift occurs during the first half hour of detector operation suggests that it should be possible to obtain a significant improvement in the rate of initial drift by suitable pre-treatment of the electrodes. The effect of pretreating perforated foil electrodes by immersion in a substrate solution, containing 3×10^{-5} M of thiocholine methane-sulphonate, for several hours before use is shown in Table 5 and Figure 7. Electrode potentials were again measured relative to a standard calomel electrode.

TABLE 5

EFFECT OF ELECTRODE PRETREATMENT ON BASELINE DRIFT

Running Time (min)	Drift in Electrode Potential (mV)		Total Baseline Drift (mV)
	Anode	Cathode	
0	0	0	0
2	80	35	115
5	90	45	135
10	90	55	145
15	90	70	160
20	100	80	180
30	90	80	170
40	90	80	170

Examination of the rates of initial baseline drift given in Table 5 indicates that pretreatment of the electrodes results in a marked reduction in the time required for both anode and cathode to achieve their stable operating potentials. One method of obtaining a similar improvement with clean electrodes is to reduce the effective surface area of the electrodes. The effect of using a small ring of platinum wire, laid horizontally on the surface of the enzyme pad (Figure 3b), for the cathode and a piece of platinum wire gauze for the anode, is shown in Table 6.

TABLE 6

EFFECT OF ELECTRODE DESIGN ON BASELINE VOLTAGE

Running Time (min)	Baseline Voltage (mV)		
	Foil Cathode/ Foil Anode (Total Surface Area = 140 sq.mm)	Ring Cathode/ Foil Anode (Total Surface Area = 88 sq.mm)	Ring Cathode/ Gauze Anode (Total Surface Area = 73 sq.mm)
0	80	390	330
10	183	412	335
20	230	428	335
40	260	445	335
60	272	455	340
80	282	463	340

Exposure of the detector to GB vapour (concentration approximately 10 mg/m^3) using the ring cathode/foil anode or ring cathode/gauze anode electrode system yielded exactly the same response time (5 sec) as observed when using perforated foil for both anode and cathode.

Long Term Operation

Under clean air conditions, enzyme pads were used in the detector for periods of up to 22 hours without any measurable loss in enzymic activity.

Response to agents during long term running was investigated by operating the detector continuously for 72 hours and inhibiting the enzyme pads with GB vapour (concentration approximately 10 mg/m^3) at random intervals. Table 7 lists the results observed using a ring cathode/gauze anode electrode system.

TABLE 7

LONG TERM OPERATION

Running Time (hr)	Initial Baseline (mV)	Final Baseline (mV)	Drift (mV/min)	Response Time (sec)	Signal (mV)
4	330	430	0.42	6	175
18	410	530	0.11	6	75
5	500	540	0.14	5	75
18	520	560	0.04	5	65
5	525	550	0.08	5	70
22	520	570	0.04	5	65

The stability of the substrate solution was studied separately. Substrate solution (2×10^{-4} M) was stored for 48 hours at 25°C, both under nitrogen and in an open vessel. The rate of production of thiocholine under the two storage conditions is shown in Table 8.

TABLE 8

STABILITY OF SUBSTRATE SOLUTION

Storage Time (hr)	Thiocholine Concentration (μ mole/l)	
	Under Nitrogen	In Air
0	4	4
6	5	5
12	11	6
24	25	8
36	51	10
48	100	12

Examination of Table 8 shows that the concentration of thiocholine in the substrate solution increased from 4×10^{-6} M to 8×10^{-6} M over a 24 hour period in the presence of air. The effect of such an increase on the hydrolysed substrate baseline would be an 8 mV drift over 24 hours operation and a depression of 20 mV (8%) in the signal obtained by complete inhibition of the enzyme pad.

Storage Stability of Reagents

(i) Enzyme

Table 9 shows the results obtained by storage of the enzyme polymer (activity 22 IU/g) for a period of one month at temperatures of 25, 38 and 50°C. Preliminary experiments have shown that the stability of the enzyme polymer is unaffected by incorporation into paper.

TABLE 9

STORAGE OF ENZYME POLYMER

Storage Time (days)	Loss in Activity (%)		
	25°C	38°C	50°C
3	0	2	7
7	0	5	15
14	0	7	21
28	0	7	31

(ii) Substrate

The stability of butyrylthiocholine methane-sulphonate has previously been determined (8) and found to be satisfactory for Service use.

DISCUSSION

The results obtained upon exposure of the detector to known concentrations of G-agent vapour (Table 2) show that the system is capable of responding to agent concentrations as low as 0.005 mg/m^3 in under 5 minutes and to high agent concentrations ($\geqslant 10$ mg/m^3) in five to eight seconds. Extremely high sensitivity is undoubtedly a desirable feature for a continuously operating automatic warning device but, unfortunately, the cumulative nature of the inhibition process means that the detector will also eventually respond to agent concentrations below the detoxification level (ca. 0.005 mg/m^3). Further, continuous exposure to such low concentrations of agent will result in a gradual rise in the normal operating potential towards the inhibited pad baseline and could result in a situation where there is insufficient potential difference available for the detector to respond to the presence of a significant agent concentration. The first of these problems, the elimination of undesirable response to low agent concentrations, could be overcome by using suitable electronic circuitry to programme the detector to respond only to a certain rate of change of signal. It should not be difficult to set such a value since the rate of agent response (Table 2) decreases rapidly at concentrations below 0.01 mg/m^3. The second problem, the gradual rise in baseline voltage, is a more difficult one but one solution would be to incorporate a malfunction circuit into the alarm logic, so that the enzyme pad is automatically changed when the potential difference across the electrodes exceeds a certain value.

Loss in enzymic activity by physical dissolution from the pad is not a problem with the chemically insolubilised cholinesterase used in this work and enzyme pads have been run continuously in the detector for periods up to 22 hours without any detectable loss in activity. The effect on enzymic activity of prolonged exposure of the detector to normal battlefield contaminants has not yet been examined, but no problems have been experienced with the ordinary laboratory atmosphere.

Although the response to V-agent vapour has not been investigated there is no reason to suppose that the detector performance will be appreciably different from that observed in the presence of G-agents. Similarly, aerosol sampling should not present any untoward problems since the enzyme paper is physically comparable to the special silica gel paper used in the Residual Vapour Detector (RVD) which retains 70 - 75% of 0.3 μ particles.

One of the most difficult problems encountered during the course of these experiments has been the development of a satisfactory method for the delivery of the air/liquid mixture to the surface of the top electrode in the detector cell. Various procedures have been attempted including pre-mixing of the air and liquid, liquid flowing down the inside of a tubular cathode or the outside of a spiral cathode and a wire gauze cathode attached to the end of the liquid delivery tube. The only satisfactory arrangement, however, has been that shown in Figure 1 and this is not totally acceptable since it requires precise assembly and the narrow bore liquid delivery tube makes the system extremely sensitive to small changes in the liquid flow rate. The constancy of the liquid flow rate has a large effect on the amplitude of the baseline "noise" produced by the electrodes which, in turn, directly affects the sensitivity and speed of response of the system. Thus the development of an improved air/liquid delivery system should enable an improvement in performance to be achieved and as evidence of what is realistically attainable, the present system has, under optimum conditions, responded to high concentrations (approximately 10 mg/m^3) of agent in 2 - 3 seconds.

Confirmation that the detector operating parameters such as air flow, liquid flow and substrate concentration in relation to cholinesterase activity, have been optimised is demonstrated by the results obtained from the evaluation of baseline voltage

in terms of thiocholine concentration (Table 3; Figure 5). Approximately 15% ($\equiv 3 \times 10^{-5}$ M) of the substrate is hydrolysed by the enzyme under the present operating conditions and this represents a virtually optimum value in terms of baseline stability and speed of response. Decreasing the concentration of hydrolysed substrate from this value would result in the baseline being exceedingly sensitive to small changes in temperature or flow rate, while increasing the concentration of hydrolysate would slow down the response.

A major part of this study has been devoted to an examination of electrode design and to the effect of the electrodes on response time, signal size and baseline drift. Individual measurement of the anode and cathode potentials has confirmed previous observations (3) that approximately 90% of the measured signal is due to a change in anode potential and it has been shown that both electrodes contribute to the baseline drift (Table 4). The high rate of initial baseline drift which was experienced using perforated platinum foil electrodes (Table 6) has been largely overcome by using a small loop of platinum wire for the cathode and a piece of platinum wire gauze for the anode. The rate of baseline drift with the latter electrode assembly (0.1 mV/min) can be easily compensated for electronically without affecting the sensitivity of the detector since, even in the worst case of G-agent response (0.005 mg/m^3 GA), the rate of signal increase was 5.5 mV/min.

The effect of prolonged continuous operation upon detector performance has been examined for periods of up to 72 hours duration and agent responses have been shown to be satisfactory, with the exception that the size of the total signal obtained gradually diminishes (Table 7). The reason for the latter is that the thiocholine baseline drifts towards the iodine oxidation potential and so the potential difference available for the signal becomes less. This problem could be overcome by omitting iodide ion from the substrate solution so that the level of the unhydrolysed substrate baseline is no longer artificially depressed. The possibility of operating the detector under these conditions is currently being investigated.

A very disturbing feature observed, upon occasion, during the course of this study has been sudden shifts of 50 - 100 mV in the potential of the hydrolysed substrate baseline. The cause of these large voltage changes has not yet been fully determined but the most probable explanation is that the anode becomes coated with a surface layer of oxide which leads to excessive polarisation of the electrode. One possible solution to this problem seems to be to use a much higher applied current (ca. 10 μA) and preliminary results in this direction are very promising. However, should the latter prove unsuccessful, it may be necessary to resort to the use of graphite electrodes which have been reported (9) as being particularly suited to the measurement of electrochemical oxidation processes and which do not suffer from the formation of surface oxide films.

CONCLUSIONS

1. Immobilised cholinesterase can be used to provide a rapid and extremely sensitive method for the automatic detection of G-agent vapour. It is expected that the method will be applicable to all anticholinesterase agents.

2. The use of cholinesterase covalently bonded to a special polymer has overcome the difficulties associated with leaching of the enzyme from the detector cell during continuous operation.

RECOMMENDATIONS

1. The performance of the system towards a wide range of anticholinesterase agents, in both vapour and aerosol form, should be examined.

2. The electrode chemistry and the cell design should receive further study to enable a reliable instrument to be developed for field use.

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APPENDIX I

ASSAY OF ENZYME PADS

REAGENTS

Buffer   0.05M phosphate, pH 7.5.

Substrate   1-Methyl-5-n-butyryloxyquinolinium
              iodide, 5×10^{-3} M, in water.
              Solution usable for one day only.

Enzyme   Cholinesterase (E.C.3.1.1.8),
              1 IU/ml, in 0.05M phosphate buffer,
              pH 7.5. Solution stored at 3°C.

CALIBRATION GRAPH

Place the desired volume of enzyme solution (0.05 to 0.5 IU) in a 1 cm photometer cell, dilute to 4 ml with buffer and bring temperature of solution to 25°C. Add 1 ml of substrate solution, stir for five minutes and measure the absorbance of the solution at 462 nm. Correct for substrate solvolysis by repeating the procedure with a mixture containing 4 ml of buffer and 1 ml of substrate solution. Obtain absorbance values for five different quantities of enzyme and plot enzyme activity against absorbance.

ENZYME PADS

Macerate the pad in 4 ml buffer and bring the temperature; of the mixture to 25°C. Add 1 ml of substrate solution, stir for five minutes, filter and measure the absorbance of the filtrate at 462 nm. Correct absorbance for substrate solvolysis and obtain enzyme activity from the calibration graph.
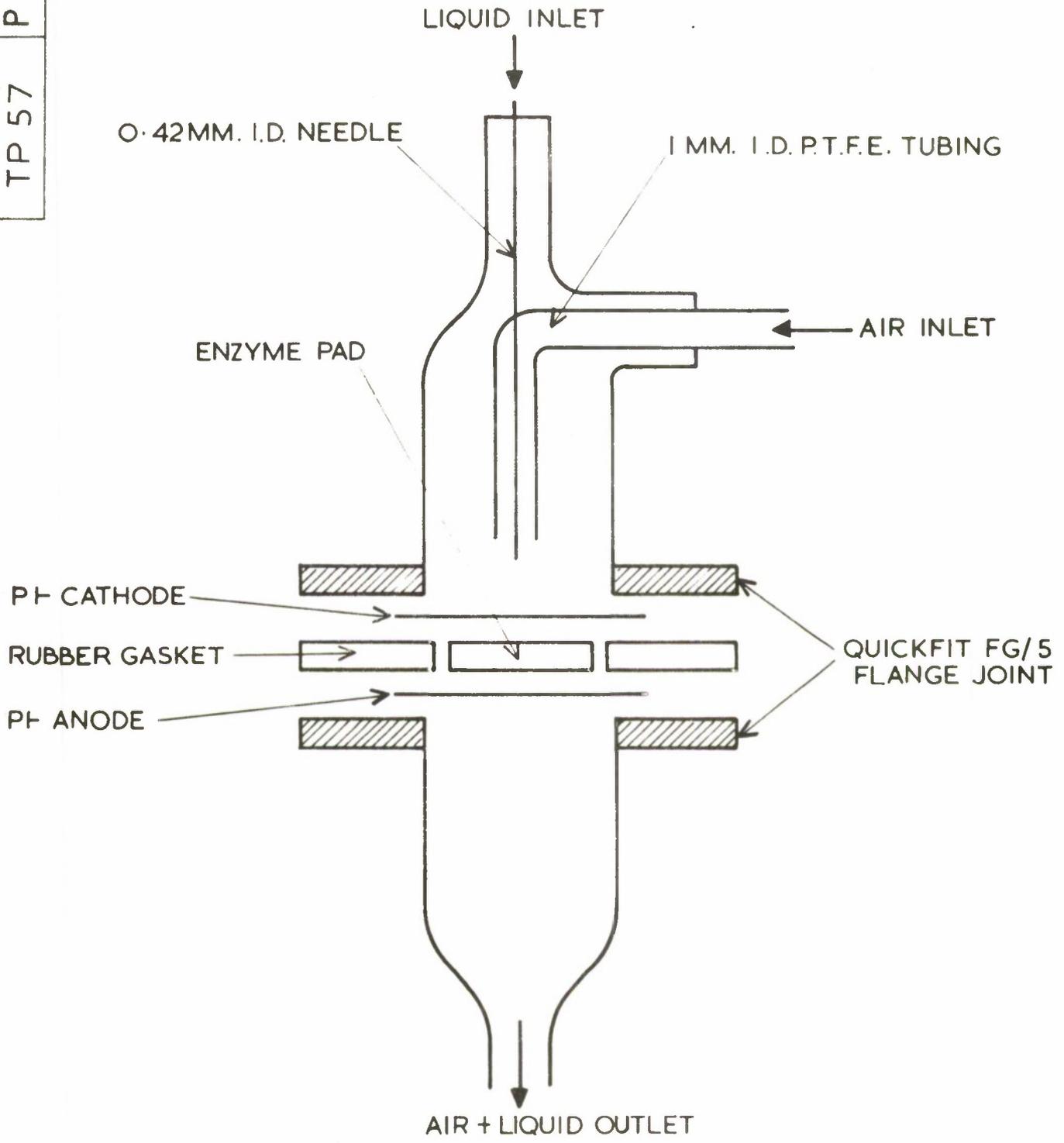
LIQUID INLET

O·42MM. I.D. NEEDLE

I MM. I.D. P.T.F.E. TUBING

ENZYME PAD

AIR INLET

PH CATHODE

RUBBER GASKET

QUICKFIT FG/5
FLANGE JOINT

PH ANODE

AIR + LIQUID OUTLET

ELECTROCHEMICAL DETECTOR CELL

FIG.I.

AIR INLET
1 MM. I.D. P.T.F.E. TUBING.

LIQUID INLET
0·42 MM.I.D. NEEDLE

LOCKING RING

Pt CATHODE

RUBBER GASKET

ENZYME PAD

Pt ANODE

AIR + LIQUID OUTLET

ELECTROCHEMICAL DETECTOR CELL

FIG. 2.

TP 57 P 3643

I MM. I.D. PERFORATIONS
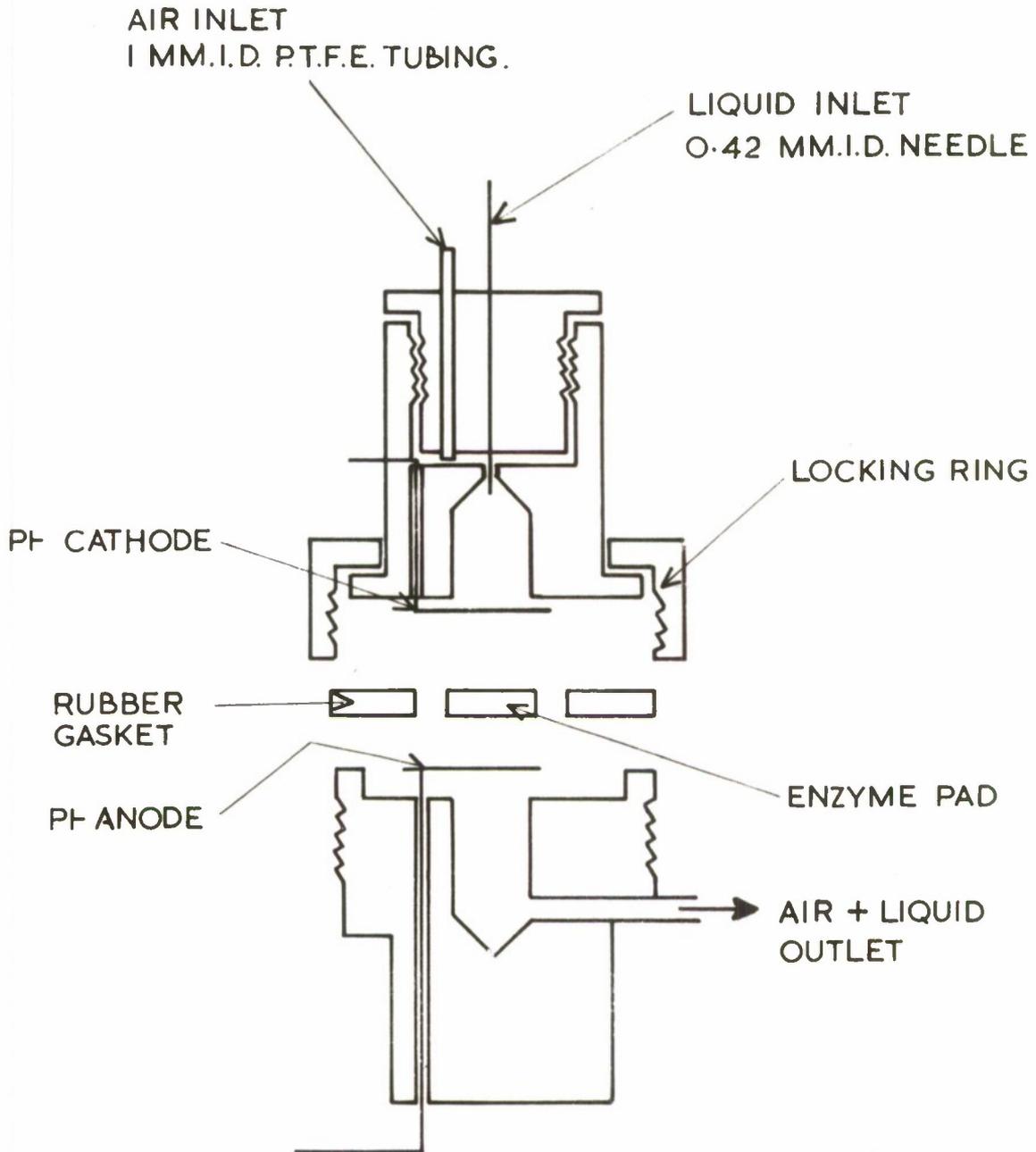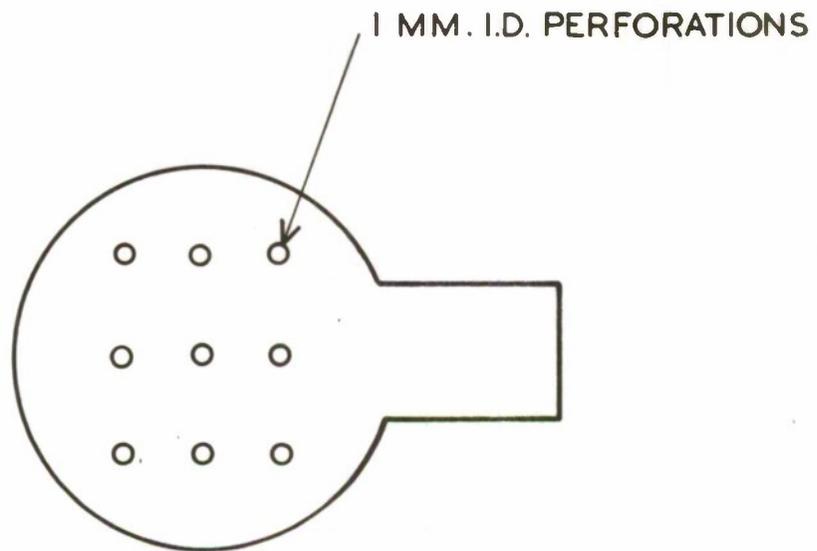
(a) PERFORATED FOIL ELECTRODE

ENZYME PAD

Pt WIRE
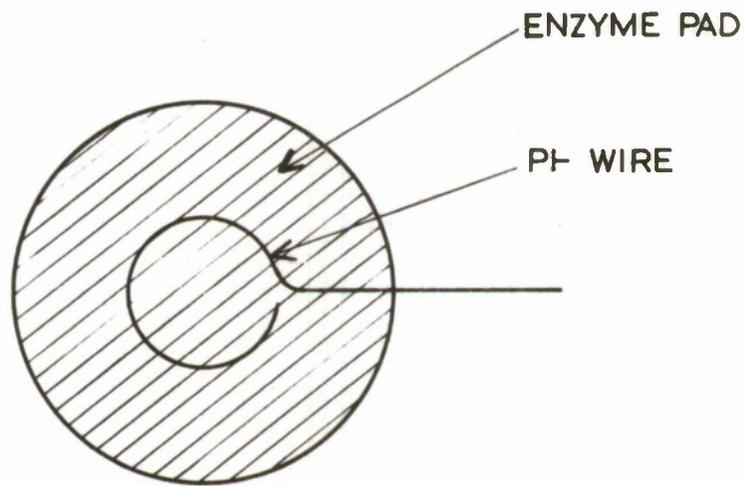
(b) WIRE LOOP ELECTRODE

PLATINUM ELECTRODES

FIG. 3.

TP 57 | PT 5899
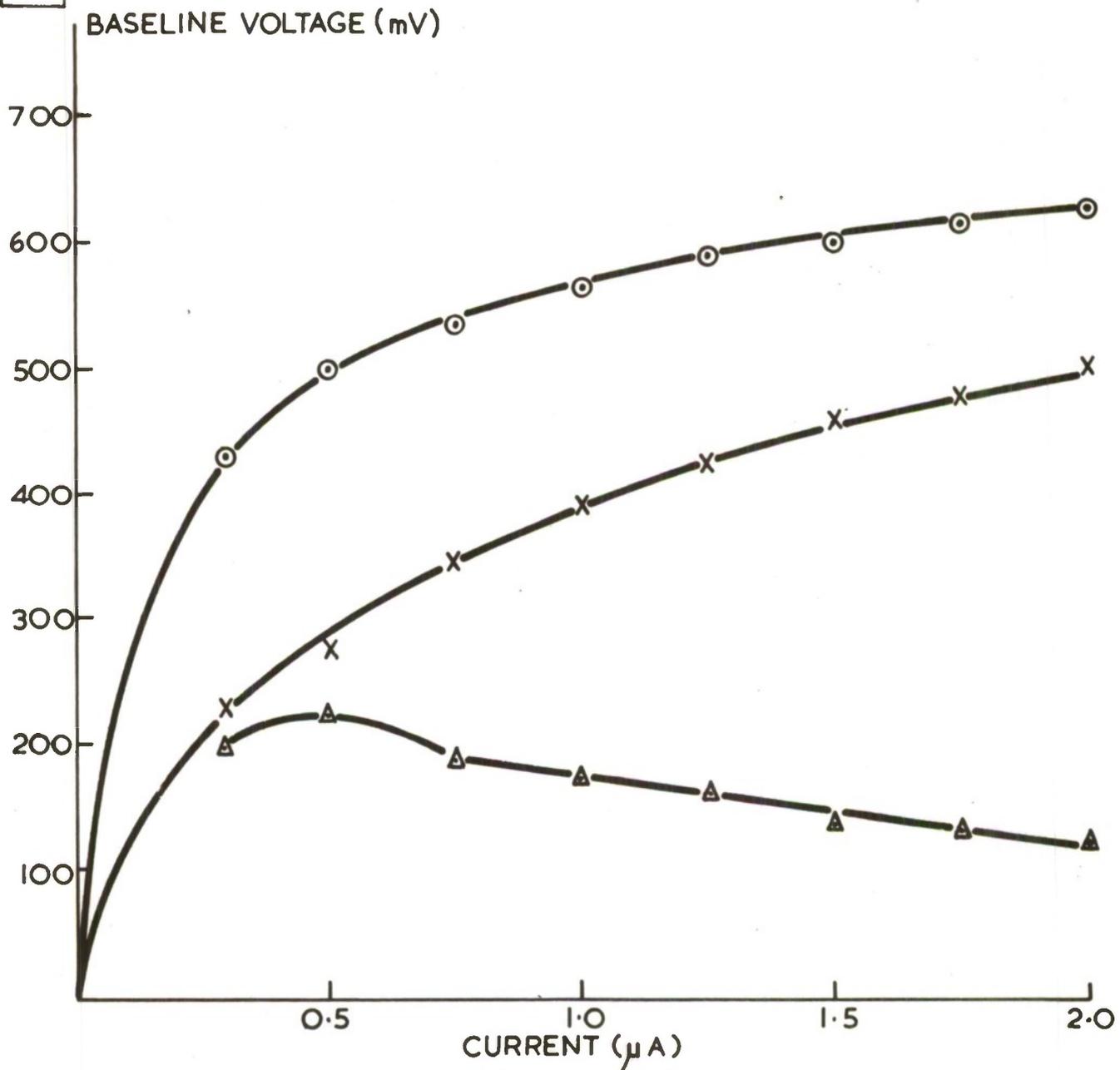
DETERMINATION OF OPTIMUM APPLIED CURRENT

FIG.4.

PT 5900

TP 57

- - - - DETECTOR OPERATING RANGE IN CLEAN AIR.

BASELINE VOLTAGE (mV)
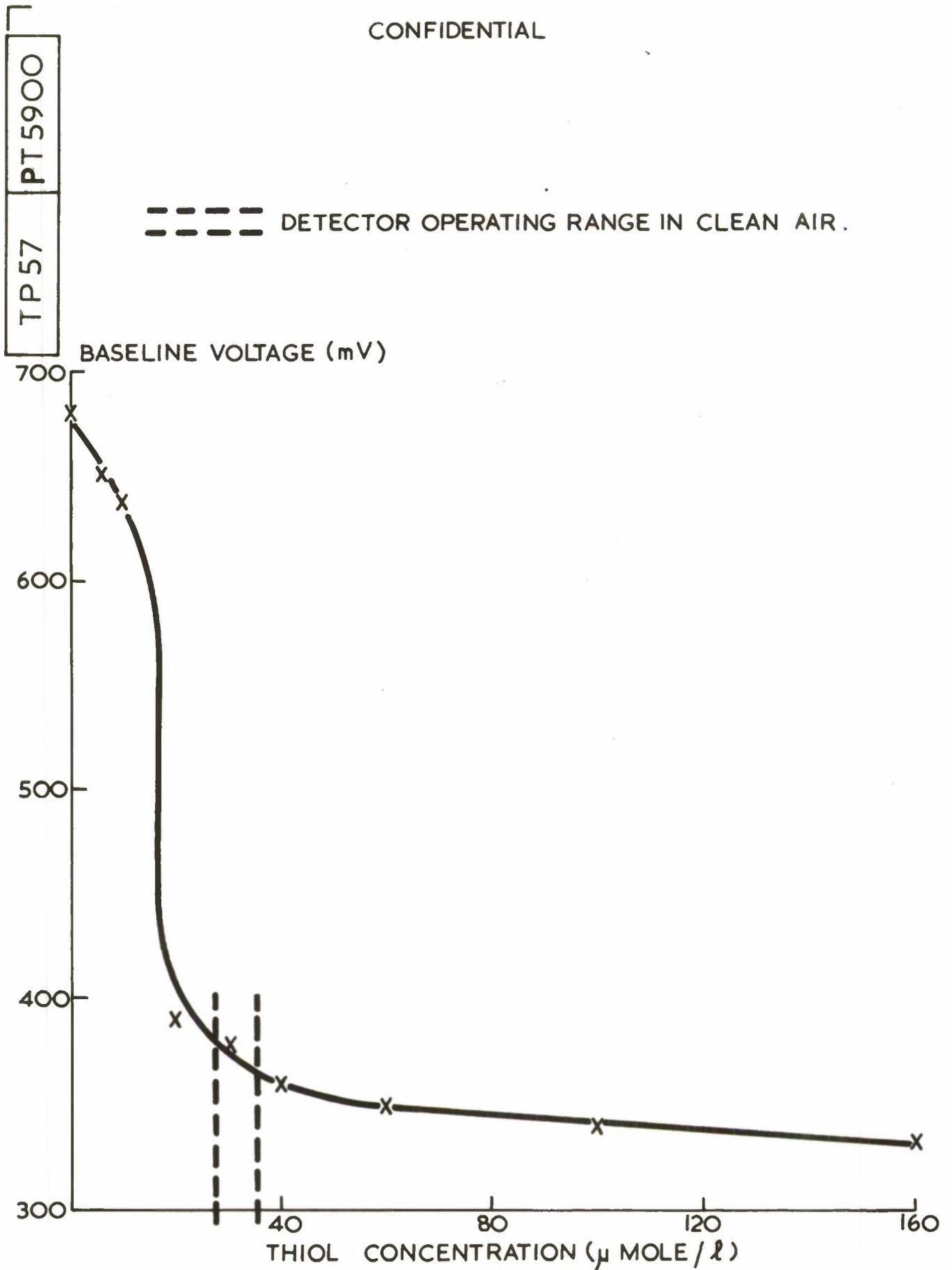
EFFECT OF THIOCHOLINE CONCENTRATION ON BASELINE VOLTAGE

FIG. 5.
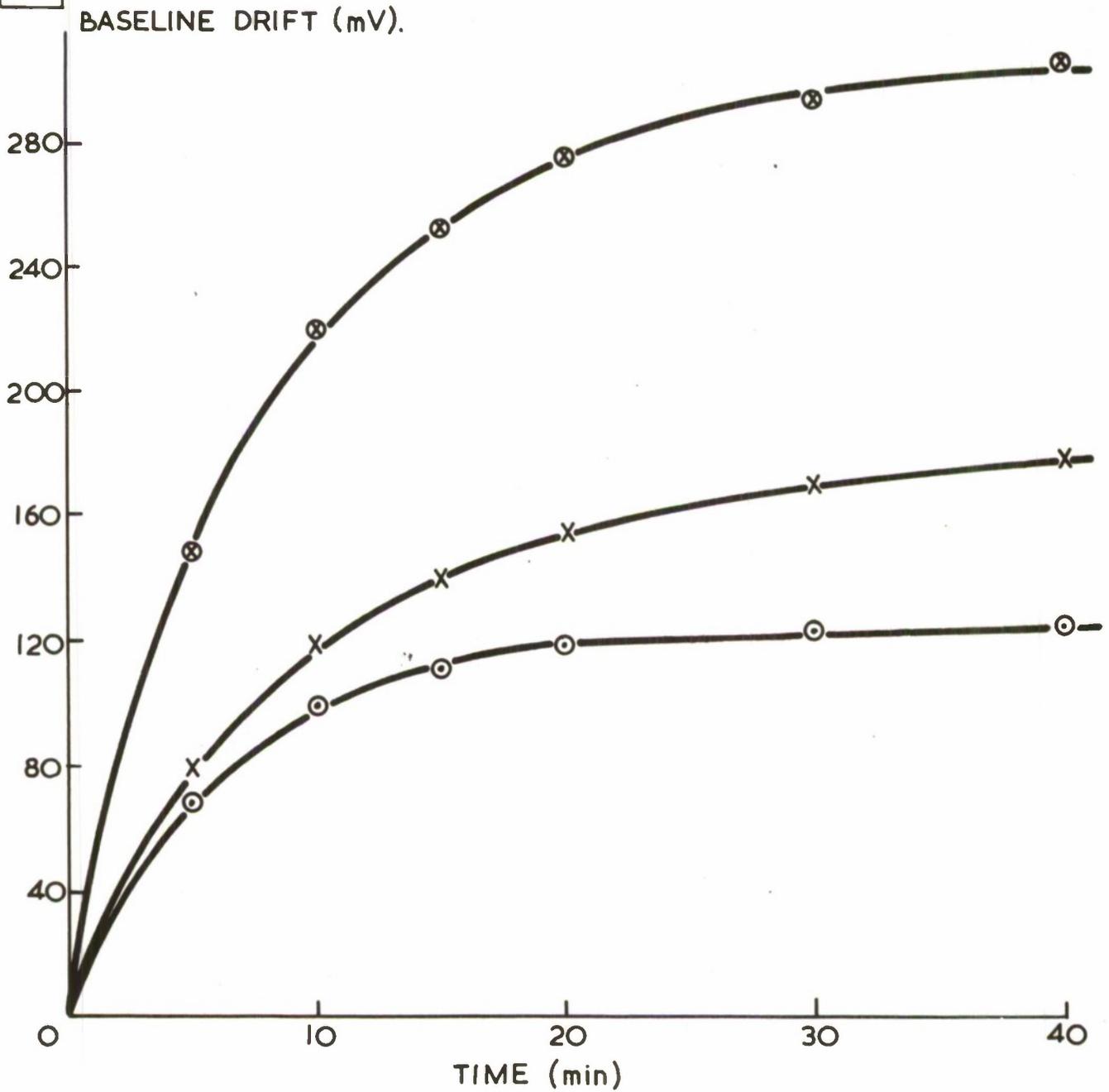
EFFECT OF ANODE AND CATHODE ON BASELINE DRIFT

FIG. 6.

PT 5902

TP 57

EFFECT OF ELECTRODE PRETREATMENT ON BASELINE DRIFT.

FIG. 7.

TP 57 | PT 5903
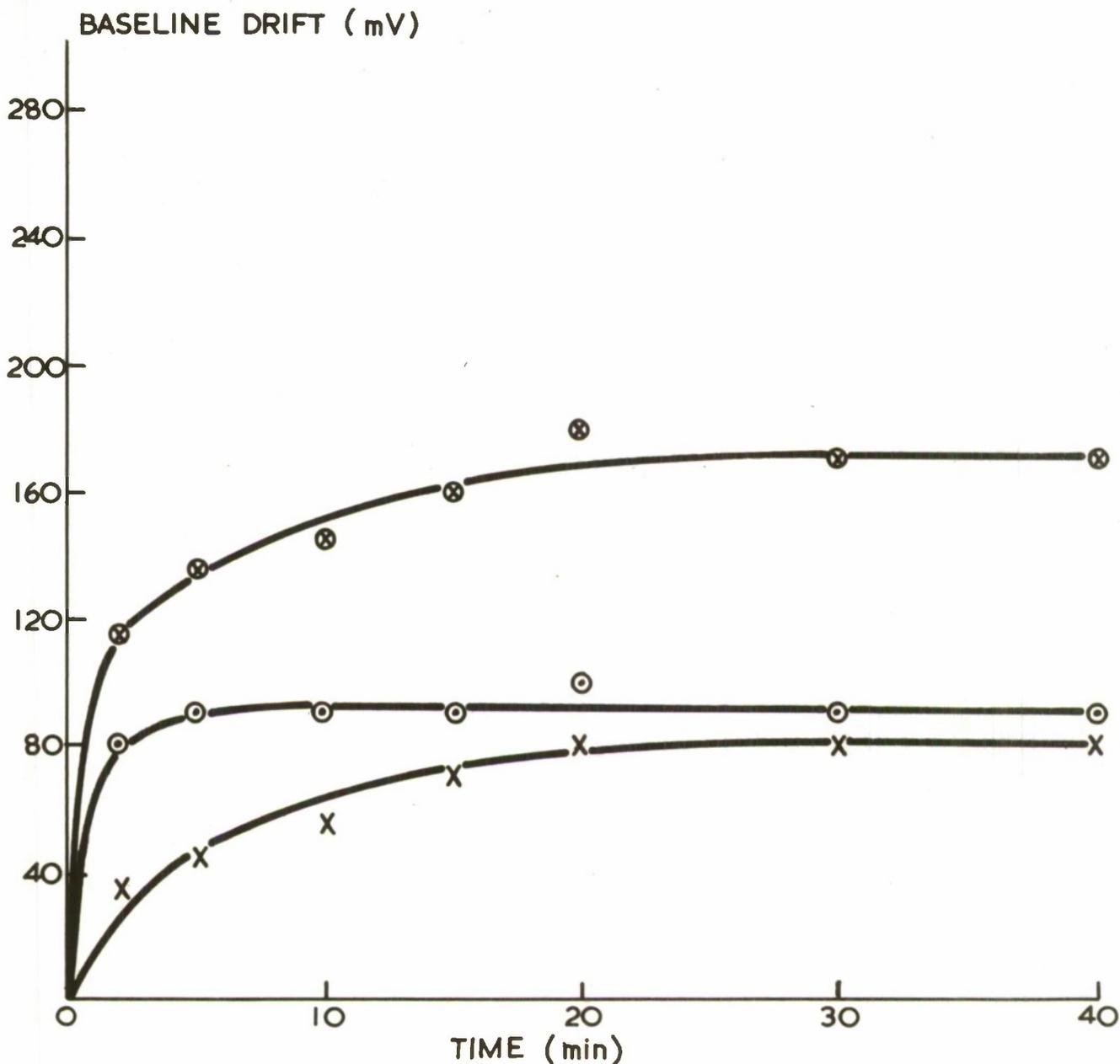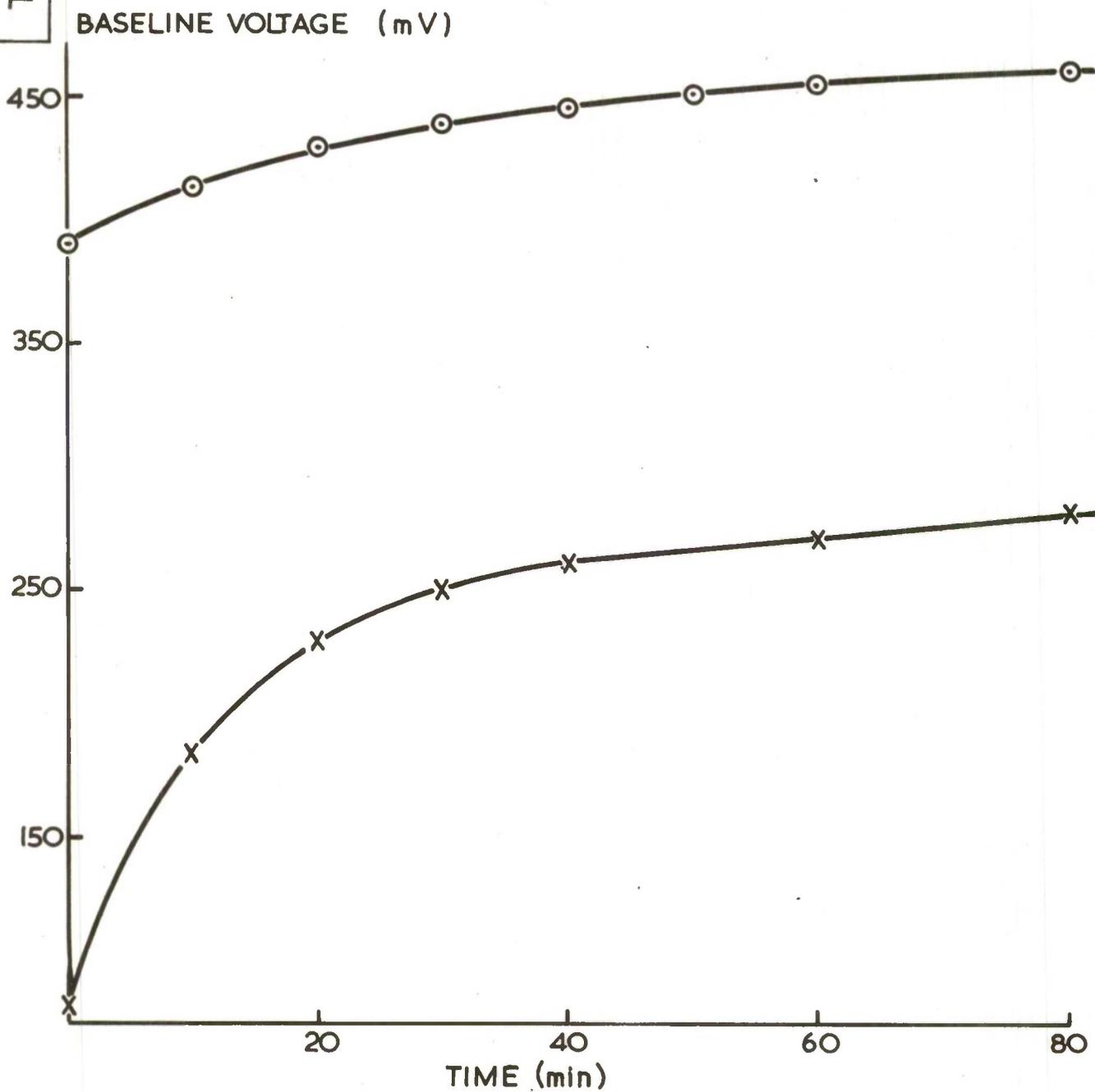
EFFECT OF CATHODE DESIGN ON BASELINE VOLTAGE

FIG. 8.

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